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Key words: allergen; Cry j 1; Cry j 2; Japanese cedar pollen; pollinosis.

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Since Mosmann & Coffman revealed the development of CD4+ T-cell subsets with distinct cytokine production profiles and functional characteristics (1), a number of studies have reported that Th2 cells producing interleukin (IL)-4 and IL-10 are generated in allergic responses, while Th1 cells producing IL-2 and interferon-gamma (IFN-γ) are induced by bacterial and viral infection (2, 3). However, how and why T cells differentiate into differing T-helper phenotypes remains unknown. Recent studies have shown that a conserved, transmembrane, envelope peptide of various retroviruses, CSK-17, induced a Th2-like response (4), whereas a leishmania antigen, LeiF, induced a Th1-like response (5). Since allergy to cedar pollen is caused by two major allergens, Cry j 1 and Cry j 2, derived from Japanese cedar pollen (*Cryptomeria japonica*), this system gives us a good opportunity to compare their molecular characteristics as allergens (6–11). Cry j 1 is localized on the exine (outside) layer (12), and Cry j 2 is contained in the amyloplast (inside) of pollen (13). Pollen extracts contain Cry j 1 and Cry j 2 at an approximate ratio of 4 : 1(14), and both allergens have been molecularly cloned

(13, 15). Cry j 1 is an enzyme exhibiting pectate lyase activity (16), and Cry j 2 has amino acid sequence homology with polygalacturonase (7). In a previous paper (6), we showed that Cry j 2 bears a limited number of immunodominant regions for T-cell immunity despite polymorphic features of HLA-DR.

In order to characterize these allergens, we examined here the cytokine production profiles of allergic subjects with peripheral blood lymphocytes (PBL) exposed to the allergens, which are more representative of the *in vivo* situation than are T-cell clones. We demonstrated

1) that T-cell proliferation assays do not necessarily reflect the level of allergenicity, although T cells might be an important target for prophylaxis of allergy
2) that Cry j 1 is the main allergen in Japanese cedar pollinosis rather than Cry j 2
3) that Cry j 1 and Cry j 2 have distinct characteristics of T-cell-stimulating activity.

Material and methods

Patients and controls

Twenty-four patients with pollinosis and six non-allergic donors were studied during the allergic season (February–May). These donors were selected on the basis of a history of seasonal allergic rhinitis, positive skin prick test, nasal provocation tests, and eosinophil staining of nasal smears. Non-allergic donors did not show any symptoms of seasonal allergic rhinitis and exhibited negative skin prick test to Japanese cedar pollen.

Purification of Cry j 1 and Cry j 2

Cry j 1 and 2 proteins were isolated from Japanese cedar pollen by affinity chromatography using monoclonal antibodies specific for either Cry j 1 (mAb 065) or Cry j 2 (mAb N26), as previously described (17, 18).

Cell culture

To examine cell proliferation in response to the allergens, PBL (6×10^4/well) were cultured for 5 days in triplicate in 96-round-bottom-well plates (3870–096, Iwaki glass, Tokyo, Japan) in 200 µl RPMI-1640 medium (ICN Biomedicals, Inc., Costa Mesa, CA, USA) supplemented with 7.5% heat-inactivated AB human serum (Biocell, Carson, CA, USA), 100 U/ml penicillin (Banyuu, Tokyo, Japan), 100 µg/ml streptomycin (Meijiseika, Tokyo, Japan), and 2-mercaptoethanol (4×10^{-5} M). The cell proliferation was determined by the addition of ^{3}H-labeled thymidine (0.5 µCi per well, Amersham, Bowling Green, St Louis, MO, USA) during the last 18 h of culture, and incorporation was measured by liquid scintillation. To examine the cytokine production profiles, cultures were performed at a cell concentration of 1×10^6/well in 24-well plates (3820–024, Iwaki glass, Tokyo, Japan) either for 16 h to isolate RNA or for 48 h to harvest supernatants for cytokine ELISA assays. Cells were stimulated with Cry j 1, Cry j 2 (0.1 µM), or purified protein derivative (PPD) (0.3 µg/ml, Nippon BCG, Tokyo, Japan) throughout experiments (6). IL-2 was measured by the CTLL2 assay. Briefly, CTLL2 (5000 cells) were cultured with various concentrations of culture supernatants for 28 h in 96-well, flat-bottom plates, as described above. Pulsing of the cells with ^{3}H-labeled thymidine was performed for the last 8 h of culture.

Cytokine ELISA assays

IL-4 and IFN-γ in the culture supernatants were determined by sandwich ELISA (19). Mouse anti-human IL-4 antibody (8D4–8, Pharmingen, San Diego, CA, USA) and biotinylated rat antihuman IL-4 antibody (MP4–25D2, Pharmingen) were used as coating and labeling antibodies, respectively, for the IL-4 assay. Mouse antihuman IFN-γ antibody (mAb-IFN-γ-15, Hayashibara Biochemical Lab., Inc., Okayama, Japan) and horseradish peroxidase (HRPO)-conjugated mouse antihuman IFN-γ antibody (mAb-IFN-γ-6, Hayashibara Biochemical Lab., Inc.) were used as coating and labeling antibodies, respectively, for the IFN-γ assay. Standard curves were generated with recombinant IL-4 and IFN-γ. The lower detection limits were 16 pg/ml for IL-4 and IFN-γ.

ELISA for IgE-binding to pollen allergens, Cry j 1 and Cry j 2

The procedures for IgE ELISA were as described previously (20, 21). Briefly, affinity-purified Cry j 1 or Cry j 2 (10 µg/well) was coated onto microplates (Maxisorp U16, Nunc, Denmark), followed by the addition of serially diluted serum samples. Antihuman ε-chain conjugated with β-galactosidase (Phadezyme Kit, Pharmacia, USA) was used to detect the bound IgE-allergen complex. As enzyme reaction substrate, 1 mM 4-methylumbelliferyl β-D-galactoside was used, and the fluorescein intensity of the reaction mixture was measured on a fluorometric microplate reader (Fluoroscan, Flow Laboratory, USA). The amounts of IgE antibodies to these allergens were represented in arbitrary units (U)/ml. Units of each serum were calculated

from standard titration curves of a pooled serum. The cutoff values were 5 U/ml for Cry j 1 and Cry j 2, respectively.

Detection of cytokine mRNA by reverse-transcription polymerase chain reaction (RT-PCR)

RT-PCR was performed as described (22). Total RNA was isolated from allergen-stimulated or -unstimulated cells using ISOGEN-LS (Wako, Osaka, Japan). cDNA was prepared from 40 µl of reaction mixtures containing total RNA (400 ng), oligo-dT (50 ng; Pharmacia Biotech AB, Uppsala, Sweden), 0.75 µl of 5× buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$, 50 mM DTT; Wako, Osaka, Japan), 0.37 µl of dNTP (5 mM each dATP, dCTP, dGTP, and dTTP; Pharmacia Biotech AB, Uppsala, Sweden), 0.1 µl RNA guard (Pharmacia, USA), and 30 U reverse transcriptase (Wako, Osaka, Japan) at 42°C for 1.5 h. The cDNA was amplified by PCR using the various primers listed in Table 1. All of the cytokine primers were designed so as to bind to sequences in different exons, such that priming of genomic DNA would yield products of distinct sizes. PCR amplification was carried out in a mixture of 4 µl of cDNA and 21 µl of PCR reaction mixture containing 2.5 µl of 10× reaction buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$; TaKaRa, Osaka, Japan), 2.5 mM dNTP (TaKaRa, Osaka, Japan), 0.1 µCi of α-^{32}P-dCTP (HAS, Hungary), and 2 U Taq polymerase (TaKaRa, Osaka, Japan), 0.1 µM of each primer. The mixture was subjected to DNA amplification with a gene thermal cycler (DNA thermal cycler 480, Perkin Elmer, CA, USA) set at 94°C for 30 s, 55°C for 1 min, and 72°C for 30 s for a total of 22–33 cycles. To standardize the cytokine gene expression among the samples, the β-actin transcript in each sample was assayed. The number of PCR cycles was determined by the amount of specific amplified product, which was increased approximately logarithmically to keep reactions within nonlimiting conditions and yet obtain detectable signals, 30 cycles for IFN-γ, IL-2, IL-10, IL-12 p35, and IL-12 p40; 33 cycles for IL-4; and 22 cycles for β-actin. Amplified products were electrophoresed on a 1.5% agarose gel. The intensity of autoradiographic bands was determined by a densitometer (Image Master, Pharmacia Biotech AB, Uppsala, Sweden). In the case of IL-12, Southern blot analysis was carried out with the 710-bp EcoR I fragment of phIL-12 p35 as an IL-12 p35 probe, or the 930-bp Apa I fragment of phIL-12 p40 as an IL-12 p40 probe, since RT-PCR generated amplified DNA bands of variable length. Both plasmid clones were obtained by PCR according to the method of Gubler et al. (23).

Table 1. Oligonucleotides used as PCR primers

Gene	Primer	Sequence	Length (bp) amplified fragment
IFN-γ	Sense[1]	5'-CAGGTCATTCAGATGTACCG-3'	380
	Antisense[2]	5'-TGGGATGCTCTTCGACCTCG-3'	
IL-2	Sense	5'-CAACTCCTGTCTTGCATTGC-3'	524
	Antisense	5'-AGGCCTGATATGTTTTAAGTGG-3'	
IL-4	Sense	5'-ATGTGCCGGGAACTTTGTC-3'	483
	Antisense	5'-AATATTCAGCTCGAACACTTTG-3'	
IL-5	Sense	5'-AGAGCCATGAGGATGCTTC-G-3'	596
	Antisense	5'-TAGTCTCAACTTTCTATTATCC-3'	
IL-10	Sense	5'-ACTTGCAAAAGAAGGCATGC-3'	564
	Antisense	5'-ACCCTGATGTCTCAGTTTCG-3'	
IL-12 p35	Sense	5'-ACATGCTCCAGAAGGCCAGAC-3'	501
	Antisense	5'-GACAGAGTGACGAGCTATCTGA-3'	
IL-12 p50	Sense	5'-TGCTGGTGGCTGACGACAATC-3'	309
	Antisense	5'-CACATTCCTACTTCTCCCTGAC-3'	

[1] Coding sequence of 5'-end.
[2] Complementary sequence of 3'-end.

Results

Relationship between pollinosis, T-cell proliferation, and concentrations of serum IgE specific to Cry j 1 and Cry j 2

In order to characterize the level of T-cell sensitization to Cry j 1 and Cry j 2, respectively, PBL were stimulated with affinity-purified Cry j 1 or Cry j 2 *in vitro*. Twenty-four patients with pollinosis and six nonallergic donors were tested for T-cell proliferation and/or IgE antibody titers specific to Cry j 1 and Cry j 2 during the spring season at Kagoshima, Japan (Table 2).

In response to Cry j 1 stimulation, 95% of allergic donors (21/22) showed T-cell proliferative responses at a stimulation index (SI) of more than 3.0. Similarly, 81% (18/22) responded to Cry j 2 at an SI of over 3.0. Subjects who showed a high SI in response to Cry j 1 stimulation appeared also to exhibit higher responses to Cry j 2. The magnitudes of both Cry j 1 and Cry j 2 responses were at almost the same level, with a mean SI value of 11. Thus, both Cry j 1 and Cry j 2 show comparable immunopotencies in inducing T-cell proliferation. In the case of nonallergic donors, T cells from two donors were highly responsive to Cry j 1 and Cry j 2, while T cells of four other nonallergic donors showed weak responses at an SI of 1.6–3.9. Therefore, it appears that T cells activated by either Cry j 1 or Cry j 2 in allergic and nonallergic subjects might be distinct, and that this does not simply correlate with allergic states.

Among the subjects examined, 17 allergic donors were tested for the amount of Cry j 1- and Cry j 2-specific IgE antibodies in their sera. Anti-Cry j 1 IgE antibody was produced in 100% of these

Table 2. Serum IgE and T-cell proliferation in response to Cry j 1 and Cry j 2

	Proliferation (SI)[1]				IgE (U/ml)[2]	
Donors	Cry j 1	*P* value[3]	Cry j 2	*P* value[3]	Cry j 1	Cry j 2
Allergic						
M.H.	32.6	<0.01	15.1	<0.05	133	120
D.T.	29.8	<0.005	33.8	<0.001	26.8	0[4]
Y.I.	19.8	<0.001	28.6	<0.01	n.d.	n.d.[5]
E.Y.	18.5	<0.02	15.5	<0.01	n.d.	n.d.
K.H.	17.5	<0.05	22.7	<0.001	n.d.	n.d.
A.Y.	14.1	<0.001	15.7	<0.025	n.d.	n.d.
J.T.	13.9	<0.025	6.4	<0.001	221	0
M.D.	11.3	<0.01	14.7	<0.001	n.d.	n.d.
F.N.	8.8	<0.05	24.6	<0.001	108	124
M.Y.	7.6	<0.05	2.4	<0.10	366	9.6
K.O.	7.4	<0.02	14.7	<0.005	101	25.9
H.H.	7.4	<0.005	5.5	<0.025	109	0
K.S.	7.3	<0.001	10.5	<0.005	41.7	0
T.I.	7.0	<0.005	5.2	<0.001	n.d.	n.d.
M.K.	6.5	<0.005	13.5	<0.01	n.d.	n.d.
M.S.	5.0	<0.005	4.1	<0.02	465	8.3
M.O.	4.2	<0.10	8.0	<0.005	92.4	69.6
K.K.	3.1	<0.05	1.2	<0.95	852	42
S.N.	3.0	<0.001	5.9	<0.01	32.7	0
Y.M.	3.0	<0.001	2.0	<0.01	84.6	13.4
H.S.	3.0	<0.01	1.8	<0.50	42	22
M.E.	2.5	<0.025	4.9	<0.025	17.4	15.8
K.B.	n.d.		n.d.		63.9	6.1
S.Y.	n.d.		n.d.		57	0
Mean	10.6		11.7		165.5	26.9
Nonallergic						
S.H.	14.9	<0.01	16.5	<0.05	0	0
H.A.	8.6	<0.005	12.8	<0.001	0	0
Y.K.	3.9	<0.20	3.3	<0.20	n.d.	n.d.
T.M.	2.9	<0.005	3.9	<0.05	n.d.	n.d.
N.T.	1.7	<0.30	2.6	<0.10	n.d.	n.d.
T.H.	1.6	<0.30	3.1	<0.30	n.d.	n.d.
Mean	5.6		7.0		0	0

[1] SI (stimulation index): allergen-stimulated response/nonstimulated response. Mean value of background response (nonstimulated) was 927 ± 85 dpm (disintegrations per minute) for 22 allergic subjects and 754 ± 100 dpm for six nonallergic subjects. Lowest background response was 183 ± 59 dpm for M.H. and highest was 2445 ± 615 dpm for H.S.

[2] Defined by Hashimoto et al. (Ref. 20) and Sakaguchi et al. (Ref. 21).

[3] Two-tailed Student's *t*-test: allergen-stimulated response vs. nonstimulated response.

[4] Below detection limit (5 U/ml).

[5] n.d.: not determined.

donors at a mean value of 165 U/ml. In contrast, anti-Cry j 2 IgE antibody was detected in only 64% of the allergic donors at low levels with a mean value of 26 U/ml. Thus, in contrast to T-cell proliferation, Cry j 1 exhibited dominant immunopotency in IgE antibody production in comparison with Cry j 2. Furthermore, there appeared to be no correlation of response levels between T-cell proliferation and IgE antibody production.

It was noted that two (S.H. and H.A.) of six nonallergic donors without allergic symptoms did not produce IgE antibodies specific to Cry j 1 and Cry j 2 despite showing remarkable T-cell proliferation. The other nonallergic donors exhibited neither a T-cell response nor an anti-Cry j 1 and 2 IgE antibody response (skin prick test). Thus, T-cell activation by Cry j 1 and Cry j 2 does not necessarily lead to either induction of IgE production or elicitation of pollinosis.

Cytokine production profiles

In order to characterize the T cells responding to Cry j 1 and Cry j 2, PBL were stimulated with these allergens *in vitro*, and the amounts of IFN-γ and IL-4 in the culture supernatants were measured 48 h later by ELISA (Table 3). All allergic subjects examined (16/16) significantly produced IFN-γ in response to Cry j 1 with a mean value of 934 pg/ml. On the other hand, in response to Cry j 2, only five allergic subjects (K.S., F.N., M.D., M.Y., and A.Y.) showed remarkable elevation in IFN-γ production, with a mean value of 679 pg/ml. The mean value of the amount of IFN-γ produced by nonstimulated cells was 23 pg/ml. The average SI in Cry j 1- and Cry j 2-stimulated IFN-γ responses among the 16 allergic subjects were 39 and 13, respectively. Thus, Cry j 1 showed dominant IFN-γ-inducing properties in comparison with Cry j 2.

IL-4 was not detected in any of the samples by ELISA under these experimental conditions. In the same experiments, nine subjects were tested for IL-2, IFN-γ, IL-4, and IL-10 mRNA expression by RT-PCR using RNA extracted from cells 16 h after stimulation. Representative results shown in Fig. 1 and Table 4 summarize these data. IFN-γ gene expression was comparable to the data obtained by ELISA (Table 3). Cry j 1 induced a relatively higher expression of the IL-10 gene at an average SI of 9.0 than Cry j 2 with a mean SI of 1.2. Thus, Cry j 1 was again predominant as an inducer of IL-10 as well as inducing IFN-γ in comparison with Cry j 2. IL-4 gene expression was elevated to a similar level in both Cry j 1- and Cry j 2- stimulated cells. IL-4 gene expression was not detected in any of the nonallergic subjects (data not shown). In the case of IL-2 gene expression, Cry j 2-stimulated cells exhibited slightly stronger expression than Cry j 1-stimulated cells. The CTLL2 assay detected IL-2 of 62.6 and 64.3 pg/ml in culture supernatants obtained from Cry j 1- and Cry j 2-stimulated cells, respectively, as a mean value of eight allergic subjects.

We also examined the expression of IL-12 p35 and IL-12 p40 genes as a cytokine produced by accessory cells favoring the generation of Th1-like responses (Table 5). The expression of IL-12 p35 was relatively constant with little influence by allergen stimulation. However, IL-12 p40 was

Table 3. Cytokine production in response to Cry j 1 and Cry j 2

	IFN-γ (pg/ml)					IL-4 (pg/ml)		
	Stimulant					Stimulant		
Donors	Cry j 1	SI[1]	Cry j 2	SI	None	Cry j 1	Cry j 2	None
Allergic								
K.S.	6850	146	109	2	47	<16	<16	<16
D.T.	2400	150	30	2	<16	<16	<16	<16
F.N.	1170	73	1520	95	<16	<16	<16	<16
M.S.	1050	66	22	1	<16	<16	<16	<16
M.O.	1000	63	1150	72	<16	<16	<16	<16
H.H.	764	48	<16[2]	1	<16	<16	<16	<16
K.B.	325	15	20	1	22	<16	<16	<16
H.S.	311	19	<16	1	<16	<16	<16	<16
M.Y.	237	5	167	3	52	<16	<16	<16
A.Y.	200	13	450	28	<16	<16	<16	<16
Y.M.	144	8	19	1	17	<16	<16	<16
J.T.	83	1	71	1	55	<16	<16	<16
S.Y.	74	5	<16	1	<16	<16	<16	<16
M.E.	32	2	<16	1	<16	<16	<16	<16
M.H.	30	2	70	4	<16	<16	<16	<16
K.K.	20	1	<16	1	<16	<16	<16	<16
Mean	918	39	232	13	23			

[1] SI (stimulation index): allergen-stimulated production/nonstimulated production.
[2] <: lower than detection limit.

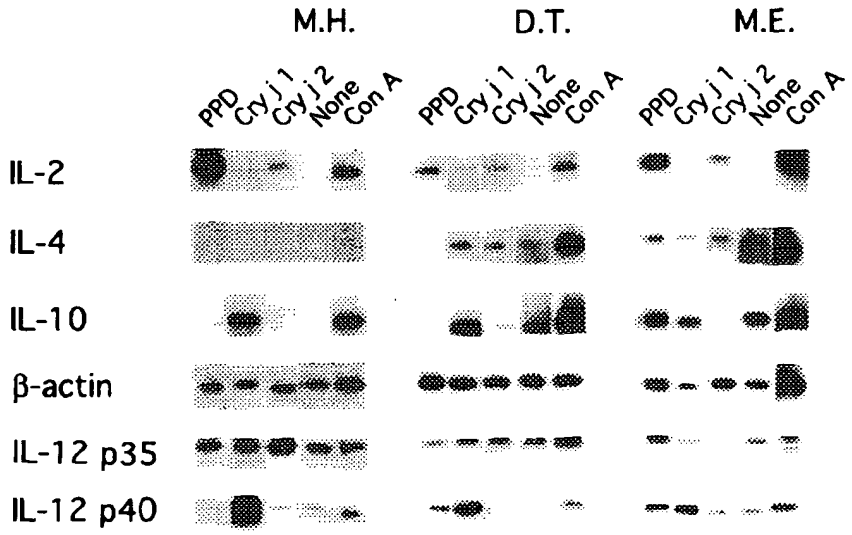

Fig. 1. RT-PCR analysis of cytokine gene expression. β-actin transcript in each sample was used to standardize various cytokine gene expressions among samples. PPD: purified protein derivative; Con A: concanavalin A.

highly elevated after stimulation by Cry j 1 in all eight cases examined. The average SI for Cry j 1 and Cry j 2 were 8.4 and 1.2, respectively. As can be seen in the ratios of Cry j 1/Cry j 2 at the level of each subject, the dominant preference of Cry j 1 for enhanced IL-12 p40 gene expression was evident in comparison with Cry j 2. All of these results are summarized in Table 6. Thus, Cry j 1 strongly induced IFN-γ, IL-12 p40, and IL-10. In contrast, Cry j 2 induced IL-2 and IL-4 comparably to Cry j 1, but only weakly induced IFN-γ, IL-12, and IL-10 in spite of its strong induction of T-cell proliferation.

We did not analyze the cytokine profiles of nonallergic subjects further, since we found only two nonallergic subjects who showed significant Cry j 1- and Cry j 2-induced T-cell proliferation.

Table 4. Th1/Th2 response profiles (RT-PCR) induced by Cry j 1 or Cry j 2 stimulation

	Th1 type						Th2 type					
	IL-2 (SI)[1]			IFN-γ (SI)			IL-4 (SI)			IL-10 (SI)		
Donors	Cry j 1	Cry j 2	1/2[2]	Cry j 1	Cry j 2	1/2	Cry j 1	Cry j 2	1/2	Cry j 1	Cry j 2	1/2
Allergic												
D.T.	>8.1[3]	>18.3	0.4	171.4	2.1	81.6	3.0	2.3	1.3	12.1	0.9	13.4
M.S.	>30.1	>35.4	0.9	65.6	1.4	46.9	3.0	2.0	1.5	1.9	0.1	19.0
K.S.	14.8	165.4	0.1	145.7	17.9	8.1	0.9	2.0	0.5	>12.3	>0.6	20.5
Y.M.	19.6	14.0	1.4	8.5	1.1	7.7	0.5	1.4	0.4	29.4	6.3	4.7
K.K.	2.2	4.6	0.5	6.7	1.3	5.2	2.1	1.2	1.8	10.1	0.5	20.2
M.E.	11.7	30.0	0.4	6.4	1.4	4.6	0.9	0.5	1.8	0.9	0.01	90.0
M.Y.	1.1	3.9	0.3	4.6	3.2	1.4	0.8	0.7	1.1	1.5	1.3	1.2
K.O.	7.1	13.2	0.5	n.d.[4]	n.d.	n.d.	0.2	0.9	0.2	4.9	>1.2	4.1
S.N.	>3.7	>9.0	0.4	n.d.	n.d.	n.d.	1.8	2.7	0.7	7.7	>0.3	25.7
Mean	>11	>33	0.5	**58.4**	4.0	**22.2**	1.5	1.5	1.0	**9.0**	1.2	22.1

[1] SI (stimulation index): radioautographic intensity of allergen-stimulated group/that of nonstimulated group.
[2] 1/2 = Cry j 1 (SI)/Cry j 2 (SI).
[3] >: In this case, nonstimulated cells exhibited no mRNA expression (no visible band of autoradiography). Therefore, comparison was made with lowest visible band of nonstimulated cells of other patient in same experiment. Comparison was made under same expression level of β-actin gene.
[4] n.d.: not determined.

Table 5. IL-12 gene expression (RT-PCR) by Cry j 1- or Cry j 2-stimulated PBL

	IL-12 p35 (SI)[1]			IL-12 p40 (SI)		
Donors	Cry j 1	Cry j 2	1/2[2]	Cry j 1	Cry j 2	1/2
Allergic						
M.S.	n.d.	n.d.	n.d.	10.7	0.2	53.5
D.T.	0.7	0.7	1.0	33.1	0.7	47.3
K.K.	n.d.	n.d.	n.d.	3.8	0.3	12.7
K.S.	0.2	0.4	0.5	3.3	0.6	5.5
S.N.	<0.1[3]	3.5	0.03	1.9	0.6	3.2
K.O.	1.1	n.d.[4]	n.d.	2.5	1.1	2.3
Y.M.	2.5	0.9	2.7	11.9	6.2	1.9
M.Y.	1.0	1.2	0.8	0.3	0.2	1.5
Mean	0.9	1.3	1.0	**8.4**	1.2	**15.8**

[1] SI (stimulation index): see Table 4.
[2] 1/2 = Cry j 1 (SI)/Cry j 2 (SI).
[3] <: In this subject, expression was not detected in Cry j 1-stimulated cells but was detected in nonstimulated cells. Therefore, SI was tentatively calculated as described in footnote of Table 4.
[4] n.d.: not determined.

Discussion

From results obtained on the serum IgE levels in 145 patients residing in the Tokyo area, it has been reported that Cry j 2 is as important a major allergen as Cry j 1 (20). In our study, all of the allergic subjects examined produced anti-Cry j 1 IgE antibody. However, 36% of them did not produce anti-Cry j 2 IgE antibody (Table 2). There are potential causes of this variation, including the fact that this study involved patients from a different environment. Particular factors in the Tokyo area could potentiate the allergenicity of Cry j 2. Otherwise, the content of Cry j 2 may differ between cedar pollens obtained from the Kagoshima and Tokyo areas. Thus, this study showed that Cry j 1 was basically the major allergen, in comparison with Cry j 2, in terms of allergenicity. In contrast, both Cry j 1 and Cry j 2 induced the same degree of T-cell proliferation. Even two nonallergic subjects showed strong T-cell proliferation in response to Cry j 1 and Cry j 2. These results suggest that T cells activated by Cry j 1 and Cry j 2 in either allergic or nonallergic subjects may be distinct, and that this activation does not necessarily lead to either induction of IgE responses or elicitation of pollinosis. A similar observation was reported by O'Brien & Thomas (24), and O'Brien et al. (25), studying T-cell responses to the house-dust-mite allergens (*Dermatophagoides pteronyssinus*) Der p 1 and Der p 2.

We could not detect IL-4 in the culture supernatants obtained 48 h after either Cry j 1 or Cry j 2 stimulation, although IL-4 mRNA was detected in these cells 16 h after starting culture. Since a number of studies have detected high levels of IL-4 production in allergic or atopic patients (26–30), our results were unexpected. We stimulated PBL at 3 μg/ml (0.1 μM) of cedar-pollen allergen, which was an optimal concentration for T-cell proliferation (6). Comparable concentrations have been shown to elevate IL-4 production in other studies on patients allergic to either Der p 2 (31) or *Lolium perenne* pollen (Lol p 1) (26). It is well known from *in vivo* experiments that exposure to low doses of antigen favors the priming of Th1-like responses,

while high doses favor the generation of Th2-like responses (32). On the other hand, recent studies found IL-4 production *in vitro* by PBL derived from allergic patients to be induced at very low concentrations of allergen (0.003–0.01 μg/ml), but not at high concentrations (10–30 μg/ml) (30). Pfeiffer et al. showed that there was an inverse relationship between IL-4 production and T-cell proliferation as regards the antigen concentration required to induce a reaction (33). Since IgE was produced at high levels in most patients (Table 2), IL-4 must be produced by PBL in these patients *in vivo*, although the contribution of IL-13 was not estimated. Thus, it is uncertain why IL-4 production was so low in our *in vitro* system.

In addition to IL-10 as a Th2-like cytokine, IL-5 gene expression was also examined in four subjects (D.T., Y.M., K.K., and S.N.) in the experiment of Table 4. Cry j 1 and Cry j 2 equally increased the IL-5 gene expression in all subjects after *in vitro* sensitization. As no IL-5 gene expression was detected in nonstimulated cells nor in PPD-stimulated cells in every case, SI values (experimental group/control group) were not presented in Table 4.

We also examined the profile of IL-12 production, as an accessory-cell-derived cytokine that acts to induce preferentially the development of Th1 cells (5, 34–37). In both allergic and nonallergic subjects, IL-12 p35 gene expression was relatively constant even after stimulation by the allergens. In contrast, IL-12 p40 gene expression was remarkably increased by Cry j 1, but not Cry j 2 (Table 5). The same observation has been reported by D'Andrea et al. (38). It is of interest that Cry j 1 shows characteristics of an IL-12 inducer, because it induces high levels of IgE production, while Cry j 2 does not. What characteristic feature of this allergen determines the ability to induce IL-12 production remains unknown. IL-10 strongly inhibits IFN-γ production by suppressing IL-12 synthesis in accessory cells (38, 39). If Cry j 2 is a potent inducer of IL-10, Cry j 2 might be a weak inducer of IL-12. However, this is unlikely because Cry j 2 hardly elevated IL-10 gene expression (Fig. 1 and Table 6).

In this context, it has been reported that a conserved, transmembrane, envelope peptide of various retroviruses, CSK-17, induces Th2-like responses (4), whereas a recombinant leishmania antigen, LeiF, induces Th1-like responses (5). These studies suggested that CSK-17 acts directly on monocytes/macrophages, enhancing production of IL-10 and reducing production of IL-12, and that LeiF functions as an adjuvant. This possibility is under investigation for Cry j 1 and 2.

Numerous studies have demonstrated that IL-12 promotes the generation of Th1 cells secreting

Table 6. Summary

Immunologic features		Mean value		
		Cry j 1		Cry j 2
IgE (U/ml)[1]		**165**	>	**26**
		(0)[5]		(0)[5]
T-cell proliferation[2]		10.6	=	11.7
		(5.6)[5]		(7.0)
Cytokines				
Th1 type	IL-2 (SI)[3]	11	≤	33
	IFN-γ (SI)[3]	**58.4**	>	4.0
	IFN-γ (SI)[4]	**39**	>	13
Th2 type	IL-4 (SI)[3]	1.5	=	1.5
	IL-10 (SI)[3]	**9.0**	>	1.2
Macrophage	IL-12 p35 (SI)[3]	0.9	=	1.3
	IL-12 p40 (SI)[3]	**8.4**	>	1.2

[1] Serum IgE.
[2] Stimulation index (SI) = allergen-stimulated response/nonstimulated response.
[3] SI from RT-PCR experiments.
[4] SI from ELISA experiments.
[5] Number in parenthesis indicates data from nonallergic subjects.
Figures in boldface indicate significantly elevated responses.

IFN-γ, which then inhibits the generation of Th2 cells (5, 40–42). Since Cry j 1 is localized on the exine (outside) layer (12) and Cry j 2 is contained in the amyloplast (inside) of pollen (13), Cry j 1 confronts the immune system first and stimulates accessory cells to produce IL-12, which differentiates Cry j 1-specific Th0-like cells into Th1-like cells producing high levels of IFN-γ. Cry j 2 stimulation might follow this first event in immediate microenvironments. In the case of cedar-pollen allergy, we could not observe the typical Th2-like responses reported in other literatures but only the mixed features of Th1- and Th2-like responses. It appears that the localization and relative amounts of allergens in pollens at sensitization *in vivo* might critically affect the generation of Th1/Th2-like responses. Thus, microenvironments seem to be crucial when and where naive Th0-like cells encounter the allergens. The allergen-induced immunomodulating activities exhibited in the response of bulk populations of T cells *in vivo* were evidently different from those of clonal T cells during *in vitro* stimulation. The characterization of allergens in their natural forms might be important to analyze the molecular mechanisms of physiologic features of allergy and to devise practical immunotherapy for allergy.

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